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Identification of SLEEPLESS, a novel sleep-promoting factor[#]

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Abstract

Sleep is an essential process conserved from flies to humans. The importance of sleep is underscored by its tight homeostatic control. Here, through a forward-genetic screen, we identify a novel gene, *sleepless*, required for sleep in *Drosophila. sleepless* encodes a brain-enriched, glycosyl-phosphatidylinositol-anchored protein. Loss of SLEEPLESS protein causes an extreme (>80%) reduction in sleep. Furthermore, a moderate reduction in SLEEPLESS protein has minimal effects on baseline sleep, but markedly reduces recovery sleep following sleep deprivation. Genetic and molecular analyses reveal that *quiver*, a mutation that impairs *Shaker*-dependent K^+ current, is an allele of *sleepless*. Consistent with this finding, Shaker protein level is reduced in *sleepless* mutants. We propose that SLEEPLESS is a signaling molecule that connects sleep drive to lowered membrane excitability.

Insufficient and poor quality sleep is an increasing problem in industrialized nations. Chronic sleep problems diminish quality of life, reduce workplace productivity, and contribute to fatal accidents (1). Although the biological needs fulfilled by sleep are unclear (2), they are likely to be important because sleep is conserved from flies to humans (3-7), and prolonged sleep deprivation can lead to lethality (8-10). Identifying mechanisms that control sleep may lead to novel approaches for improving sleep quality.

Sleep is regulated by two main processes: circadian and homeostatic (11,12). The circadian clock regulates the timing of sleep, whereas the homeostatic mechanism controls sleep need. Homeostatic pressure to sleep increases with time spent awake and decreases with time spent asleep. Homeostatic control is thought to influence sleep under normal (baseline) conditions as well as recovery (rebound) sleep following deprivation. However, the molecular mechanisms underlying homeostatic regulation of sleep remain unclear.

A powerful approach to unraveling a poorly understood biological process is to conduct unbiased genetic screens to identify novel molecules required for that process. The *Drosophila* model for sleep is well-suited for such an approach, which proved invaluable for elucidation of the molecular basis of the circadian clock. Although several *Drosophila* genes have been implicated in sleep regulation (for example, 13-15), only one of these, the gene encoding the Shaker (Sh) K^+ channel, was isolated as a result of a genetic screen (16). A mutation in this gene causes one of the shortest-sleeping phenotypes known, validating the use of screens and suggesting that control of membrane excitability is a critical requirement for

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sleep. However, the mechanisms by which sleep homeostatic inputs regulate neuronal excitability remain unknown.

Here, using a large-scale, unbiased genetic screen, we identify a novel gene, *sleepless* (*sss*), which is required in *Drosophila* for both normal baseline sleep and rebound sleep following deprivation. We find that *sss* encodes a brain-enriched, glycosyl-phosphatidylinositol (GPI)-anchored membrane protein. We also show that *quiver* (*qvr*), a mutation causing impaired *Sh*-dependent K^+ current (17,18), is an allele of *sss*, and that Sh protein level is reduced in *sss* mutant flies. We propose that SSS protein signals homeostatic sleep drive by enhancing K^+ channel activity and thus reducing neuronal excitability.

Identification of *sss*

To identify novel genes involved in sleep regulation, we carried out a forward-genetic screen for *Drosophila* mutants with reduced daily sleep. We screened approximately 3,500 mutant lines bearing transposon insertions. A histogram summarizing the daily sleep of these lines is shown in Fig. 1A. We selected for further study the mutant line with the lowest amount of daily sleep, which we named *sleepless* (*sss*). In order to homogenize the genetic background, we outcrossed this strain five times into an isogenic wild-type strain, *iso31*, a line generated specifically for use in behavioral experiments (19). As shown in Fig. 1B, both daytime and nighttime sleep are severely reduced in both male and female *sss* mutants compared to background controls. Indeed, a small percentage of *sss* flies (~9% for both males and females) exhibits no sleep at all in our assay, a phenotype never seen in control flies. To our knowledge, *sss* mutants have the most extreme reduction in daily sleep (>85% for males and >80% for females; Fig. 1C) attributable to a single gene mutation.

Despite this extreme reduction in daily sleep, waking activity (defined as activity counts/minute awake) is not significantly elevated in this mutant (Fig. 1D), suggesting that the mutant is not hyperactive when awake (20). The marked decrease in sleep amount is largely due to a sharp reduction in the duration of sleep bouts (Fig. 1E). However, decreased sleep in the *sss* mutant is also attributable in part to a significant reduction in the number of daily sleep bouts (Fig. 1F). These phenotypes are recessive in mutant animals since flies bearing one copy of the *sss* mutation behave similarly to background controls (Fig. 1, C through F).

SSS is a novel brain-enriched, GPI-anchored protein

sss mutants bear a P-element insertion (*EY04063*, which we refer to as *P1*) in the open reading frame of a novel gene designated CG33472 by the *Drosophila* Genome Project. The genomic structure of this gene consists of 2 non-coding exons and 5 coding exons, the last of which also contains a ~3.9 kb predicted 3' untranslated region (3'UTR; Fig. 2A). In addition to the original *P1* insertion line, there is a second line, which we call *P2*, bearing a transposon insertion (*f01257*) in the 3'UTR. The SSS protein is predicted to contain a signal peptide, an N-type glycosylation site, and a potential GPI attachment site (Fig. 2, B and C). SSS is well conserved in other insect species, and there is a potential *C. elegans* homolog (F31F6.8 in Wormbase, 46% similarity for amino acids 51−133), but no obvious vertebrate homologs. Nonetheless, there may be functional vertebrate homologs with conserved downstream pathways.

To characterize the SSS protein, we generated an antibody using a peptide antigen (see Materials and Methods). This antibody recognizes two bands on Western blots of wild-type head extracts that are not detectable in sss^{P1} mutant extracts (Fig. 2D), suggesting that sss^{P1} is a severe hypomorph or null allele. Since SSS contains a consensus site for N-type glycosylation, we deglycosylated proteins from head extracts and examined SSS mobility by Western blotting. Under these conditions, only a single band of a lower apparent molecular weight than the two untreated bands is detectable (Fig. 2D), indicating that SSS is glycosylated

in vivo. Since *sss* also contains a potential GPI attachment site, we next examined subcellular localization of SSS. Transfection of *Drosophila* S2R+ cells with a wild-type *sss* construct and staining with the SSS antibody under non-permeabilizing conditions reveal a subset of the SSS protein expressed on the cell surface (Fig. 2E). Treatment of the cells with phosphatidylinositol-specific phospholipase C (PI-PLC) results in severe reduction of surface expression (Fig. 2F) and release of the SSS protein into the culture medium (Fig. 2G). These results show that the SSS protein is attached to the extracellular surface of the plasma membrane with a GPI anchor and can be released by cleavage with PLC.

Using our anti-SSS antibody, we find that SSS protein levels are enriched in fly brain and head compared to body (Fig. 2H). Consistent with these findings, *sss* mRNA expression is enriched 23−42 fold in brain compared to whole fly (Adult *Drosophila* Gene Expression Atlas (21)). SSS protein levels do not cycle in a circadian fashion, nor do they change following sleep deprivation (fig. S1, A and B, see Discussion).

Genetic analysis of *sleepless*

To determine if the sleep phenotype maps to the *sss* locus, we crossed sss^{P1} to two deficiencies that remove the locus. As predicted, both deficiencies fail to complement the short-sleeping phenotype of sss^{P1} (fig. S2A and B). In order to confirm that the sleep phenotype in sss^{P1} mutants is caused by disruption of the *sss* gene, we mobilized the P-element to generate precise and imprecise excision lines. Precise excision of the P-element restores daily sleep amount in *sss* mutants to wild-type levels (Fig. 3A and fig. S2C). We also obtained an imprecise excision allele (Δ*40*) that removes part of the *sss* coding region and is likely to be a null allele (fig. S2D). Consistent with this interpretation, $sss^{\Delta 40}$ mutants produce an undetectable level of the SSS protein (Fig. 3B). Sleep in this mutant is reduced as severely as in the *P1* mutant, and the phenotype maps to the *sss* gene since the *Δ40* allele fails to complement the *P1* allele (Fig. 3A and fig. S2C).

We next tested whether expression of wild-type SSS from a transgene can rescue the sleep phenotype of sss^{P1} mutants. Daily sleep amount is fully rescued to wild-type levels in sss^{P1} mutants carrying a genomic *sss* transgene (Fig. 3C and fig. S2E). Together with deficiency and excision experiments, the rescue data provide strong evidence that disruption of the *sss* gene is responsible for the marked reduction in sleep in sss^{P1} mutants.

As described above, sss^{P2} mutants harbor an independent transposon insertion in the 3'UTR of the *sss* gene. Homozygous sss^{P2} mutant females have similar amounts of daily sleep as controls, while mutant males have slightly lower amounts of sleep than controls (Fig. 3D and fig. S2F). In contrast, sss^{P2}/sss^{P1} transheterozygous mutants have a ~30% reduction in daily sleep compared to control/sss^{P1} flies. These data suggest that the *P2* insertion is a weaker allele than the original *P1* insertion. To examine the biochemical basis of this possibility, we performed Western analysis on head lysates from mutant and control animals. As noted above, the *P1* insertion severely reduces baseline sleep and renders SSS undetectable (Figs. 2D and 3B). In contrast, the *P2* insertion, which has minimal effect on baseline sleep, causes a moderate reduction in the level of SSS protein compared to control (Fig. 3E). Finally, transheterozygous sss^{P1}/sss^{P2} animals, which exhibit a ~30% reduction in sleep, have a greatly reduced, but still detectable level of SSS protein. These data suggest that the amount of daily sleep is correlated with the level of SSS protein and that large reductions of SSS protein are necessary to cause a significant change in daily sleep.

Reduced homeostatic response in *sss* mutants

We next sought to determine whether *sss* mutants have defects in their homeostatic response to sleep deprivation. We did not observe rebound sleep in sss^{P1} flies, but sss^{P1} flies do not

have much sleep to deprive. Thus, we tested sss^{P1}/sss^{P2} transheterozygous animals, which still have moderate amounts of sleep, as well as sss^{P2} homozygotes, which have essentially normal amounts of sleep. Mechanical stimulation results in equivalent sleep loss in sss^{P2} homozygous flies and controls and in moderately reduced sleep loss in sss^{P1}/sss^{P2} flies compared with controls (Fig. 4A and fig. S3A). Whereas control flies show substantial rebound sleep following deprivation, sss^{P1}/sss^{P2} animals have little or none (Fig. 4B and fig. S3B). Unexpectedly, a similar lack of rebound sleep is observed in sss^{P2} homozygous flies. In addition, upon lights on, control animals go to sleep faster following deprivation, but this effect is significantly less pronounced or non-existent in sss^{P2} and sss^{P1}/sss^{P2} mutants (Fig. 4C and fig. S3C).

Although other genes have been suggested to play a role in homeostatic regulation of sleep, assessment of rebound sleep in animals bearing mutations in these genes is often confounded by concomitant reductions in baseline sleep (13,16,22,23). The amount of rebound sleep generally increases with sleep lost (24,25). Thus, when comparing the effects of sleep deprivation in animals with different amounts of baseline sleep (which leads to loss of different amounts of sleep), it is unclear whether rebound sleep should be compared in absolute terms or relative to amount of sleep lost. We have circumvented this problem by studying the contribution of SSS to sleep homeostasis using the sss^{P2} mutant. The finding that sss^{P2} animals exhibit markedly reduced rebound sleep, but minimally affected baseline sleep, provides strong evidence that sleep homeostasis is impaired in these mutants.

Effect of *sss* on other behaviors and longevity

To further characterize *sss* mutants, we examined several other behavioral phenotypes. Since mutations in certain central clock genes cause baseline and rebound sleep phenotypes (9, 26-29), we analyzed the circadian rhythm phenotypes of *sss* mutants. Whereas sss^{P1} mutants exhibit weak rhythms, almost all sss^{P1}/sss^{P2} transheterozygous mutants, which display a roughly 30% reduction in daily sleep time, are rhythmic (Fig. 5A and B, Supplementary Table 1). Furthermore, daily oscillations in the level of PERIOD (PER) protein in the ventral lateral neurons (clock cells) remain intact in sss^{P1} mutants (Fig. 5C), suggesting that the reduced behavioral rhythmicity seen in these mutants is not due to a defect in the central clock.

Several other behaviors that we tested also appear normal. We find that the phototactic responses of sss^{P1} mutants are similar to those of controls (fig. S4A), and sss^{P1} mutants perform as well as controls in a taste discrimination assay (fig. S4B). sss^{P1} flies (n=43) do not exhibit a bang-sensitive paralytic phenotype, while 89% (n=56) of *easily shocked* (eas^1) flies used as a positive control do. On the other hand, the sss^{P1} mutants appear somewhat uncoordinated, and fewer mutants are able to climb a specific distance in given times compared to controls (fig. S4C). It is noteworthy, however, that despite their apparent difficulties with coordination, sss^{P1} mutants spend more time walking than controls and are capable of flying and mating. Consistent with the widely-held view that sleep serves essential biological functions, sss^{P1} mutants also exhibit a shortened lifespan compared with background controls (Fig. 5D and fig. S5).

sss is allelic to *qvr* and affects Sh expression

Because two short-sleeping mutants, *Sh* and *Hyperkinetic* (*Hk*), exhibit ether-induced leg shaking we assayed *sss* mutants for this phenotype (16,22). We find that both sss^{P1} and sss^{P2} mutants show ether-induced leg shaking. Notably, *qvr*, a mutant for which the underlying molecular defect is unknown, also has a leg-shaking phenotype, and this phenotype has been mapped close to *sss* (17). Since *qvr* mutants exhibit impaired *Sh*-dependent K^+ current (18), identification of *qvr* as an allele of *sss* would implicate *Sh* as an effector of SSS function. Genetic and molecular analyses confirm that *qvr* is indeed an allele of *sss*. The *qvr* mutation fails to complement sss^{P1} for the leg-shaking phenotype. Similarly, after being outcrossed five

times, *qvr* mutants show a significant decrease in sleep compared with wild-type controls, and sss^{P1}/qvr transheterozygotes show a further reduction in sleep (Fig. 6A and fig. S6).

We next investigated the molecular basis of the *qvr* mutation. Reverse-transcriptase polymerase chain reaction (RT-PCR) of *sss* transcripts in *qvr* mutants produces three bands, whereas that of wild-type *sss* transcripts produces a single band (Fig. 6B), indicating splicing defects in *qvr* mutants. None of the three *qvr* bands show the same electrophoretic mobility as the wild-type control band. Sequencing of the RT-PCR products reveals altered splicing of the last intron (Intron 6) of *sss* in the *qvr* mutant (Fig. 6C). A single base change found in the intron is likely to be responsible for the defective splicing (Fig. 6D). Only one of the three *qvr* transcripts (*qvr* 2) is predicted to be in frame (resulting in an insertion of 21 amino acids), and thus has the potential to produce functional SSS protein. Western analysis of *qvr* mutants reveals a small amount of SSS with a slightly higher apparent molecular weight than wild-type SSS protein, which may correspond to the product of the in-frame *qvr* 2 transcript (Fig. 6E).

Since *qvr* mutants were shown to have severely reduced *Sh*-dependent K^+ current (18), we examined whether the Sh protein level is affected in *sss* mutants. We find that one form of Sh protein is expressed at a substantially reduced level in sss^{P1} mutants compared with wild-type flies (Fig. 6F), suggesting that the effect of SSS on Sh is at least in part through its protein expression. These results establish SSS as an important regulator of the Sh K^+ channel.

Discussion

In summary, we have identified a novel *Drosophila* gene required for homeostatic regulation of sleep under normal conditions and following sleep deprivation. Although genes have been identified that regulate sleep-wake stability and baseline sleep amount, few have been demonstrated to be important for sleep rebound (13,15,30-34). Thus, further analysis of SSS function may provide a rare opportunity to gain mechanistic insight into the homeostatic regulation of sleep.

It is worth noting that sss^{P2} animals show a moderate reduction in SSS protein and a minimal reduction in baseline sleep, but have severely reduced sleep rebound. The differential requirement for SSS protein in normal versus rebound sleep may be explained in the context of the two-process model of sleep regulation, where sleep is postulated to be controlled by the opposing influences of circadian waking drive and homeostatic sleep drive (11,35). In this context, for early-morning rebound sleep to occur, a strong homeostatic signal promoting sleep would be required to counteract a strong circadian input keeping the flies awake. At night when circadian waking drive is weaker or absent, a relatively low level of homeostatic input may suffice to allow flies to sleep. The moderate level of SSS protein in sss^{P2} mutants may be within the range where sleep is possible when a wake-promoting circadian signal is low (at night), but not when it is high (in the early morning). In contrast, sss^{P1} and $sss^{\Delta 40}$ mutants, which have undetectable levels of SSS expression, display severe reductions in both baseline and rebound sleep. In these mutants, the sleep-promoting signal may be too low to allow flies to sleep even when the circadian waking drive is weak at night.

Clues to the role of SSS at the cellular level come from our biochemical characterization of this molecule. The SSS protein is a GPI-anchored membrane protein enriched in the brain. GPI-anchored proteins can function as ligands or co-receptors and can also act as diffusible signals following cleavage of the GPI anchor (36,37). Although we are unable to detect circadian or homeostatic regulation of the total levels of SSS protein, such regulation may occur at the level of cleavage of the GPI anchor. Regulation of release is known to be controlled by time of day for other proteins that do not cycle in overall levels, such as pigment-dispersing

factor, a molecular output of clock neurons (38). Alternatively, SSS may be regulated in a subset of cells that express it, which would be undetectable on our western blots.

A potential mechanism by which SSS regulates sleep is suggested by our finding that *qvr* is an allele of *sss* and that the Sh protein level is reduced in *sss* mutants. Furthermore, *qvr* mutants exhibit markedly impaired *Sh*-dependent K^+ current at the larval neuromuscular junction (18). Thus we propose that SSS lowers membrane excitability by modulating K^+ channel expression and activity. It is striking that among thousands of mutants screened in *Drosophila*, two with the strongest sleep phenotypes affect the *Sh* K^+ channel (16) and its putative regulator, *sss*. Reduced membrane excitability may thus be a central feature of sleep. Collectively, our data suggest that SSS is a signaling molecule that links homeostatic sleep drive to neuronal excitability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and notes

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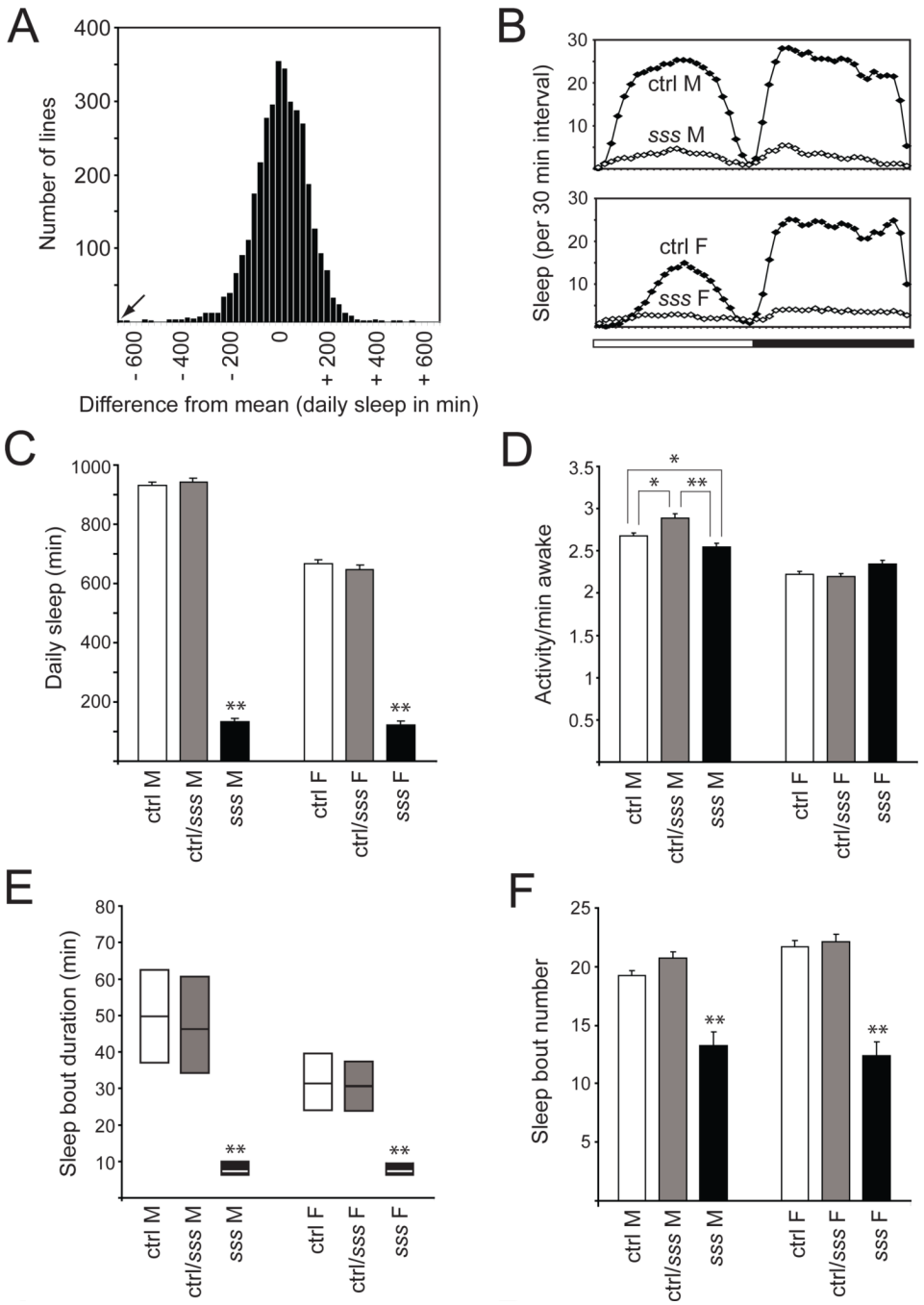

Fig. 1.

Sleep phenotype of *sss* mutants. **(A)** Histogram showing the distribution of daily sleep for ~3,500 mutant lines (~8 female flies per line). For each line, daily sleep is shown as the difference from the mean of a group of about 100−250 lines tested simultaneously. The arrow indicates the *sss* mutant line. **(B)** Sleep profile in 30-min intervals for *sss* flies (open diamonds) versus background controls (ctrl, closed diamonds). Data for male (M) and female (F) flies are shown. The bar below the x-axis indicates light (white) and dark (black) periods. **(C)** Daily sleep amount for ctrl (162 males and 148 females), ctrl/*sss* (111 males and 113 females), and *sss* flies (146 males and 148 females). Data from the same animals are shown in (C-F). **(D-F)** Activity counts/minute awake (D), sleep bout duration (E), and daily number of sleep bouts

(F) for male and female ctrl, ctrl/*sss*, and *sss* flies. In this and subsequent figures, error bars represent SEM. **P* < 0.05; ***P* < 0.0001. For (C), (E), and (F), significance level is shown for *sss* mutants compared to both ctrl and ctrl/*sss* flies. For (D), significance level is shown for pairwise comparisons as indicated by lines. In (E), sleep bout duration, which is not normally distributed, is presented as simplified box plots. The line inside each box indicates the median, and the top and bottom represent 75^{th} and 25^{th} percentiles, respectively. Approximately 9% of animals exhibiting zero sleep were excluded from calculation of sleep bout duration.

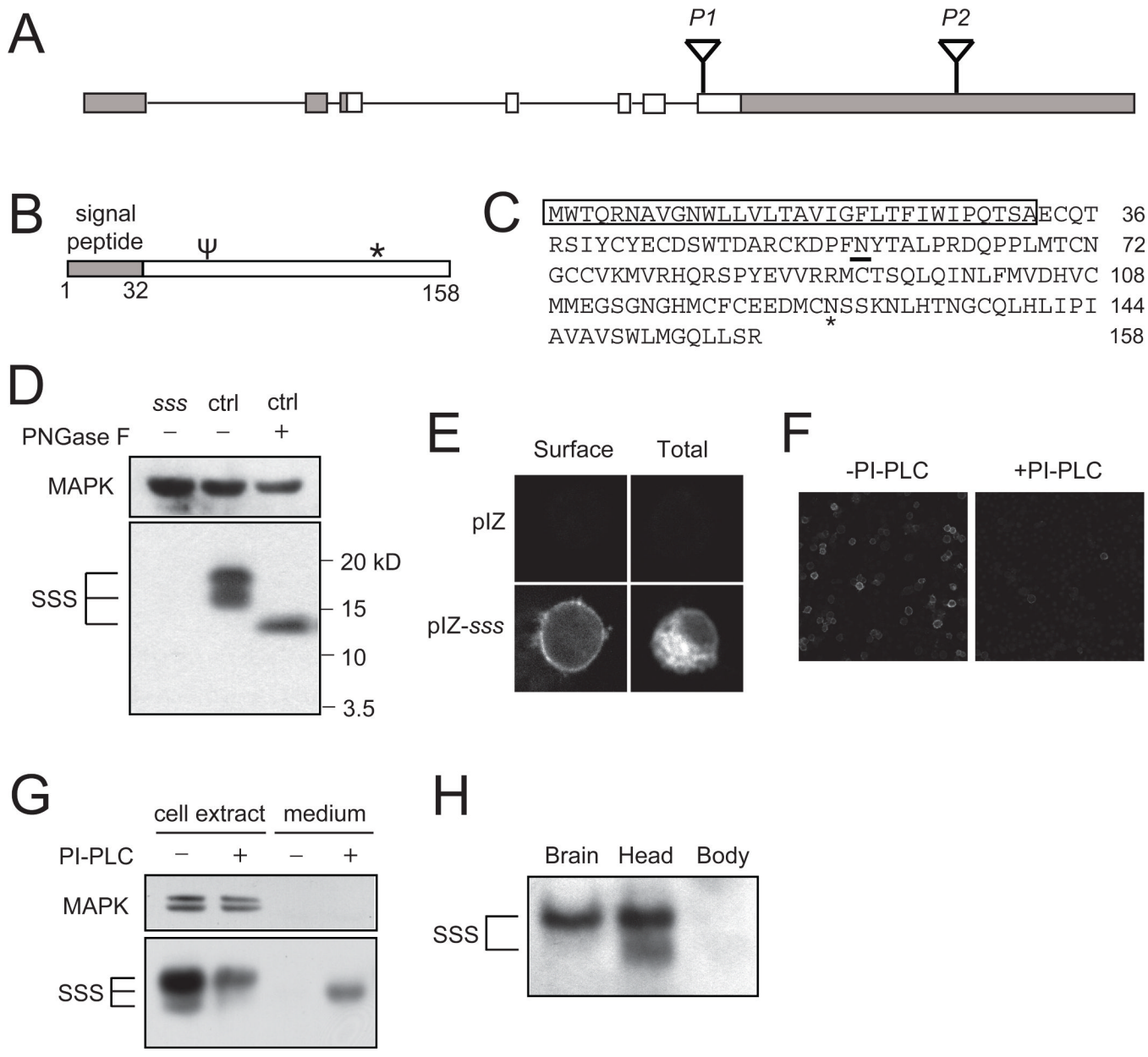

Fig. 2.

sss encodes a novel brain-enriched, GPI-anchored protein. **(A)** Schematic of the genomic structure of the *sss* locus. Non-coding regions of the cDNA are shaded, while coding regions are shown in white. **(B)** Schematic of structural features of the SSS protein. The primary sequence contains a predicted signal peptide, a N-type glycosylation site (ψ), and a potential GPI attachment site (*). **(C)** Amino acid sequence of SSS. Amino acids 1−32 comprise the predicted signal peptide (shown boxed), and the predicted N-type glycosylation site is underlined. * denotes the predicted GPI attachment site. **(D)** Glycosylation of the SSS protein. Western blot analysis with anti-SSS antibody reveals two bands detected in head extracts from wild-type (ctrl) but not *sss* flies. Deglycosylation of head extracts by treatment with PNGase F results in detection of a single band. Because our antibody to SSS does not recognize glycosylated SSS well, Western blots were treated with PNGase F before being probed with antibody to SSS. In this and subsequent Western blots, antibody to MAP kinase (MAPK) is

used to control for loading. (**E**) Surface expression of SSS in cultured *Drosophila* cells. S2R + cells were transfected with a pIZ-*sss* construct, and stained with or without permeabilization to assay for total or surface expression, respectively. Transfection with the pIZ vector alone shows specificity of our SSS antibody. (**F**) Reduced surface expression of SSS after PI-PLC treatment. S2R+ cells transfected with a pIZ-*sss* construct were stained without permeabilization after PI-PLC(+) or mock(−) treatment. (**G**) Release of SSS into the culture medium by PI-PLC. Western blot analysis of S2R+ cells transfected with pIZ-*sss* was performed after PI-PLC(+) or mock(−) treatment. **(H)** Enrichment of SSS expression in brain and head compared to body. An equal amount of total protein (~40 ug) was loaded per lane. The experiments in (D) through (H) were performed 3−4 times with similar results.

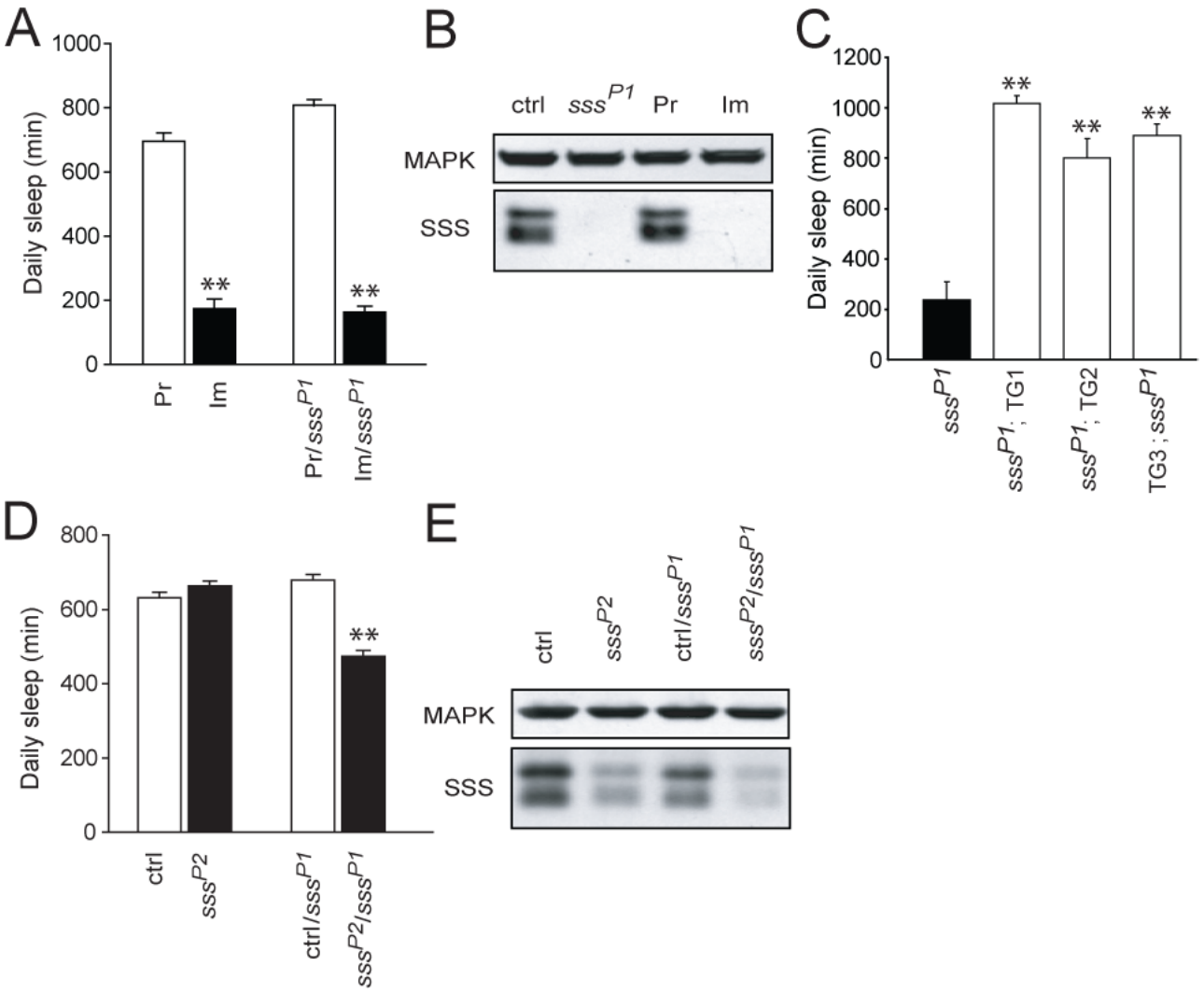

Fig. 3.

Genetic analysis of *sss*. **(A)** Daily sleep amount for precise excision (Pr, n=26), $sss^{\Delta 40}$ imprecise excision (Im, n=15), precise/sss^{P1} (Pr/sss^{P1}, n=24), and imprecise/sss^{P1} (Im/sss^{P1}, n=35) female flies. **(B)** Western blot analysis of SSS protein levels. Similar levels of SSS protein are seen in head extracts from background control (ctrl) and precise excision (Pr) flies. SSS protein is undetectable in sss^{P1} and $sss^{\Delta 40}$ imprecise excision (Im) flies. Similar results were obtained in 2 additional experiments. **(C)** Daily sleep amount for female sss^{P1} mutant flies with (sss^{P1};TG1−3, n=15,8,16, respectively) or without (sss^{P1}, n=16) a genomic *sss* transgene. TG1−3 refer to three independent transgene insertions, and either 1 or 2 copies of the transgene were present in the flies tested. **(D)** Daily sleep amount for sss^{P2} (n=110) versus background control (ctrl, n=80), as well as ctrl/sss^{P1} (n=80) versus sss^{P2}/sss^{P1} (n=112) female flies. **(E)** Reduced levels of SSS protein in sss^{P2} and transheterozygous sss^{P2}/sss^{P1} flies. Similar results were obtained in 3 additional experiments. Data from male flies of the genotypes shown in (A), (C), and (D) are available in fig S2. *$P < 0.05$; **$P < 0.0001$.

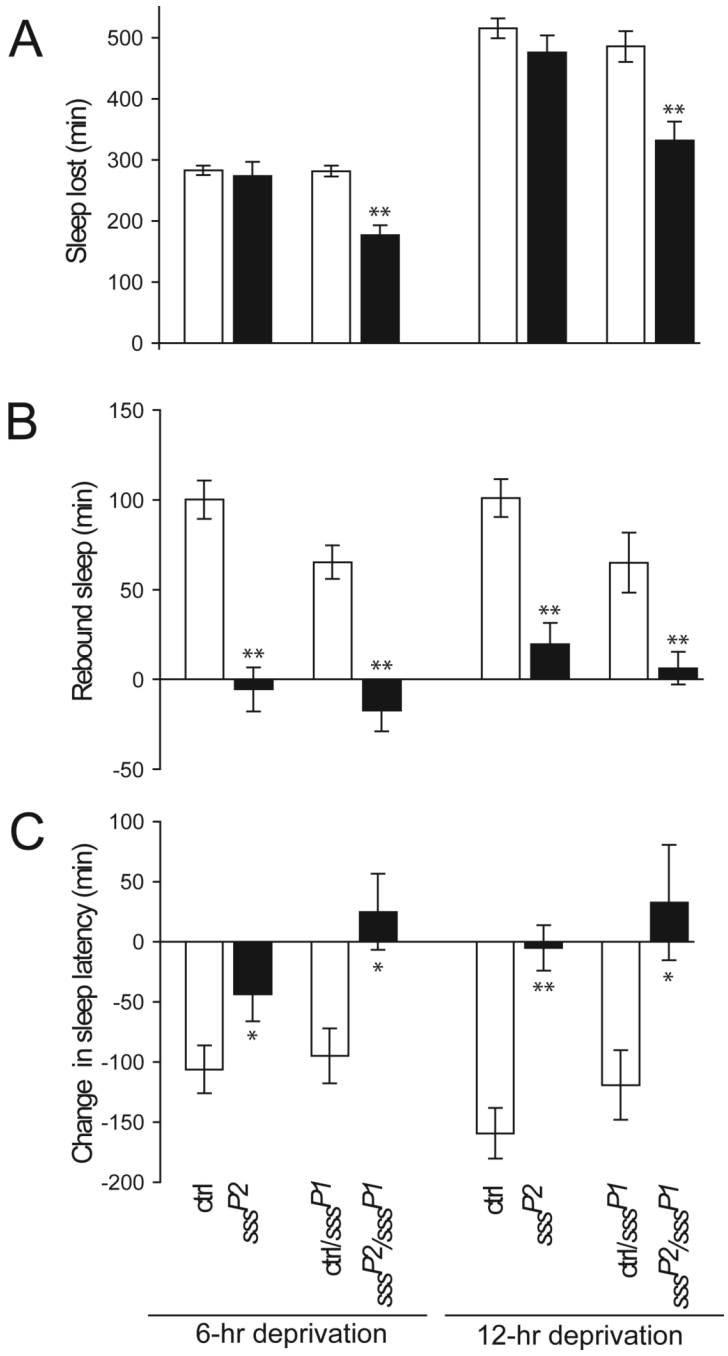

Fig. 4.

Reduced homeostatic response to sleep deprivation in female *sss* mutants. **(A)** Amount of sleep lost during 6 or 12 hours of deprivation by the end of the dark period for background control (ctrl), sss^{P2}, ctrl/sss^{P1}, and sss^{P2}/sss^{P1} flies. Data from 13−56 female flies are presented. **(B)** Amount of sleep gained during 6 hours of recovery following deprivation as in (A). **(C)** Change in sleep latency following deprivation, compared to undisturbed controls as in (A). Data from male files are shown in fig. S3. *$P < 0.05$; **$P < 0.001$.

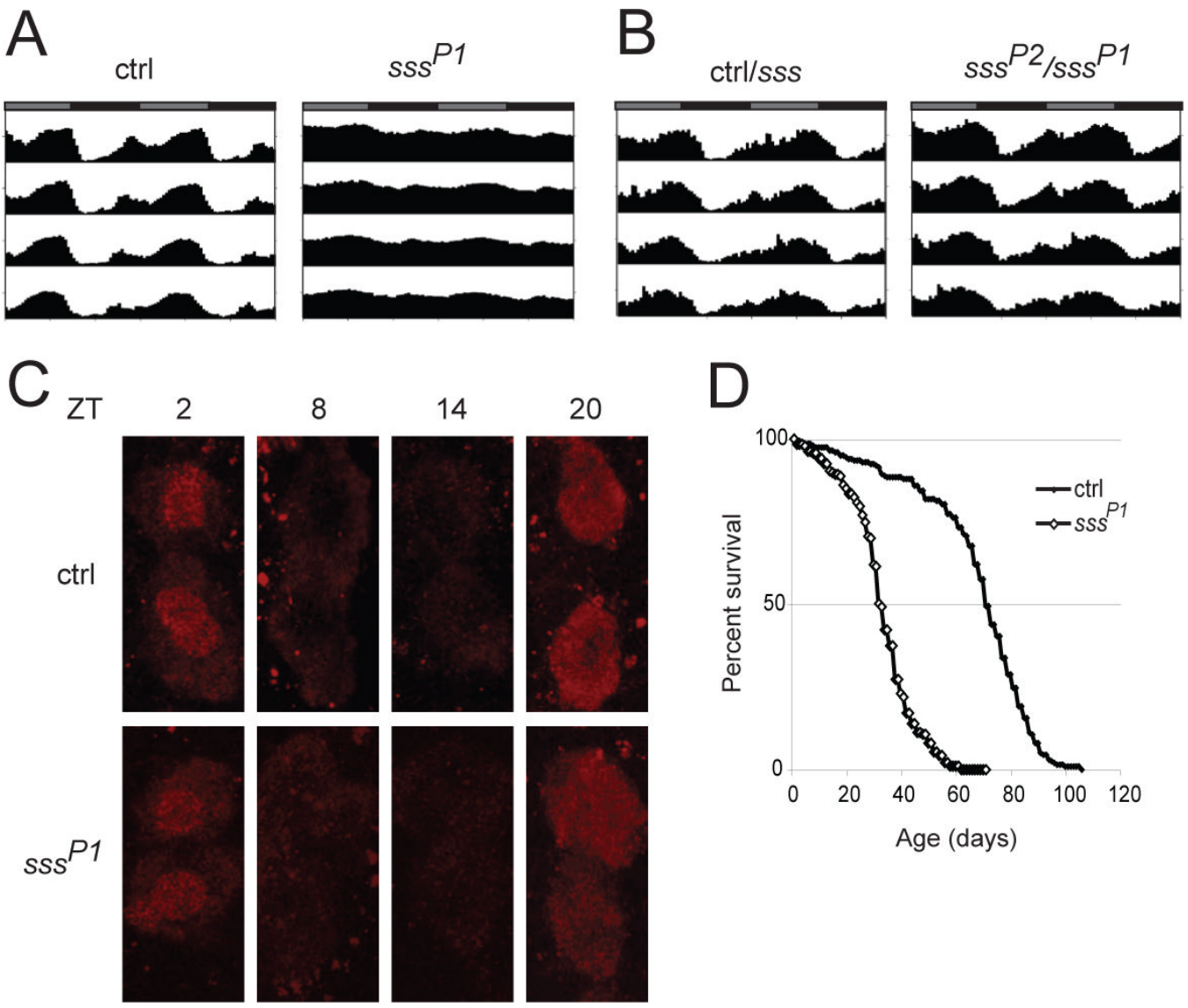

Fig. 5.

Circadian rhythm and longevity phenotypes of *sss* mutants. **(A)** Average activity records for background control (ctrl, n=64) and sss^{P1} male flies (n=81) assayed in DD. The activity records are double plotted so that each horizontal line represents data for 2 days. The gray and black bars above each activity record indicate subjective day and night, respectively. **(B)** Activity records showing average activity in DD for ctrl/sss^{P1} and ctrl/sss^{P2} (n=76) versus sss^{P2}/sss^{P1} (n=65) male flies. Circadian data for ctrl/sss^{P1} and ctrl/sss^{P2} were statistically similar and thus pooled. (**C**) Cycling of PER protein in large ventral lateral neurons in control (ctrl) and sss^{P1} mutants. Ventral lateral neurons for ctrl and sss^{P1} animals are stained for PER at indicated Zeitgeber times (ZT). PER protein level is elevated at ZT2 and ZT20, and low at ZT8 and ZT14. (**D**) Survivorship curves of background control (ctrl, closed diamonds) and sss^{P1} (open diamonds) flies. Female *sss* flies (n=187) show a significantly shorter lifespan ($P < 0.0001$) than controls (n=198). Data from male flies are shown in fig. S5.

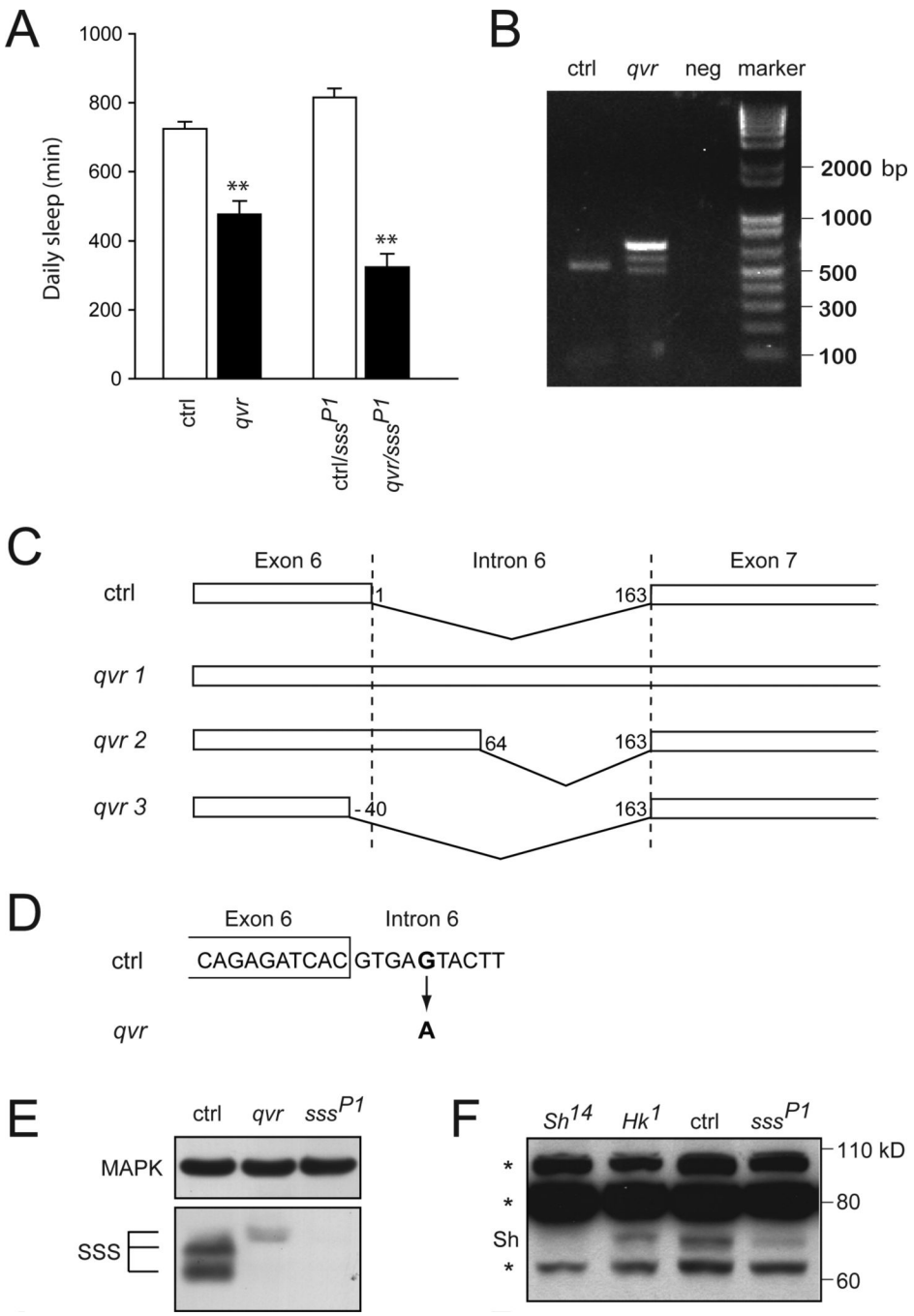

Fig. 6.

sss is allelic to *qvr* and affects Sh expression. (**A**) Daily sleep amount for *qvr* (*qvr*, n=31), versus background control (ctrl, n=32), as well as ctrl/sss^{P1} (n=30) versus *qvr*/sss^{P1} (n=32) female flies. $^{**}P < 0.0001$. (**B**) Altered *sss* transcripts in *qvr* mutants. RT-PCR products obtained with *qvr* and background control (ctrl) RNA and with water used as a negative control (neg). (**C**) Schematic representation of *sss* transcripts in *qvr* mutants. *qvr* 1, 2, and 3 correspond to the top, middle, and bottom bands, respectively. In background control (ctrl) transcripts, 163 nucleotides of Intron 6 are spliced out. In contrast, the entire intron is present in *qvr* 1 transcripts. In *qvr* 2 and 3 transcripts, splice donor sites differ from the one used in wild-type control transcripts, as indicated by the nucleotide numbers for splice sites. (**D**) Sequence change in

qvr genomic DNA in Intron 6 of *sss*. The fifth nucleotide in Intron 6 has a G to A transition. (**E**) Altered expression of SSS in *qvr* mutants. Fly head extracts from background control (ctrl), *qvr*, and sss^{P1} flies were analyzed by Western blotting with anti-SSS antibody. (**F**) Reduced expression of Sh in *sss* mutants. Western blot analysis of head extracts with anti-Sh antibody reveals a Sh-specific band that is substantially reduced in sss^{P1} mutants compared with background control (ctrl) flies. Sh^{14} flies were used to identify a Sh-specific band. Non-specific bands (*) may have obscured additional Sh bands. The experiments in (E) and (F) were performed 3 times with similar results.